S30518US

Schering AG

Hydrophilic, Thiol-Reactive Cyanine Dyes and Conjugates Thereof with Biomolecules for Fluorescence Diagnosis

This application claims the benefit of the filing date of U.S. Provisional Application Serial No. 60/443,197 filed January 29, 2003.

This invention relates to new fluorescence dyes from the class of cyanine dyes, especially indotricarbocyanines with an absorption and fluorescence maximum in the spectral range of 700 to 900 nm, a thiol-specific reactive group and three, preferably four, sulfonate groups, to an increase in water solubility as well as the production of dyes. This invention also relates to the conjugates of these dyes with biomolecules and uses thereof.

Background of the Invention

The use of light in medical diagnosis has recently gained importance (see, e.g., Biomedical Photonics Handbook (Editor: T. Vo-Dinh), CRC Press). A wide variety of diagnostic processes are found in the experimental test for application in various medical disciplines, e.g., endoscopy, mammography, surgery or gynecology. Light-based processes have a high instrumental sensitivity and make possible molecular detection and imaging of the smallest quantities of chromophores and/or fluorophores (Weissleder et al. (2001) Molecular Imaging, Radiology 219, 316-333).

Dyes that are fed to the tissue as exogenic contrast media for fluorescence diagnosis and imaging, and here in particular fluorescence dyes with an absorption and fluorescence maximum in the spectral range of 700-900 nm (diagnostic window of tissue), are of special interest for *in–vivo* use. Photons of this wavelength are comparatively little absorbed by tis-

sue and can therefore penetrate several centimeters into the tissue before the absorption process (primarily by oxyhemoglobin and deoxyhemoglobin) ends the light transport. Absorption can take place, moreover, by the fluorescence dyes that are introduced into the tissue, but that emit the absorbed energy in the form of longer-wave fluorescence radiation. This fluorescence radiation can be detected spectrally separated and makes possible the localization of dyes and the correlation with molecular structures to which the dye has bonded (see in this connection also Licha, K. (2002) Contrast Agents for Optical Imaging (Review). In: Topics in Current Chemistry – Contrast Agents II (Editor: W. Krause), Volume 222, Springer Heidelberg, pp. 1 – 31.).

To achieve a diagnostically significant differentiation between diseased structures and healthy tissue, the dye that is fed must lead to as high a concentration difference between the tissues as possible. This can be carried out based on tumor-physiological properties (blood supply, distribution kinetics, delayed removal). For molecular labeling of disease-specific structures, conjugates that consist of fluorescence dyes with target-affine biomolecules, such as proteins, peptides, and antibodies, can be used. After injection, a certain portion of these conjugates binds to molecular target structures, such as receptors or matrix proteins, while the unbonded portion is excreted from the body. In this way, a higher concentration difference and thus a greater image contrast in implementing the fluorescence diagnosis result.

Fluorescence dyes from the class of cyanine dyes fall into the category of promising representatives and were synthesized in many different structural widths. In particular, carbocyanines with indocarbocyanine, indodicarbocyanine and indotricarbocyanine skeletons have high extinction coefficients and good fluorescence quantum yields [Licha, K. (2002) Contrast Agents for Optical Imaging (Review). In: Topics in Current Chemistry – Contrast Agents II (Editor: W. Krause), Volume 222, Springer Heidelberg, pp. 1-31, and the references cited therein].

WO 96/17628 thus describes an in–vivo diagnostic process by means of near-infrared radiation. In this case, water-soluble dyes and biomolecule adducts thereof with specific photophysical, and pharmacochemical properties are as contrast media for fluorescence and transillumination diagnosis.

The synthesis of cyanine dyes with indocarbocyanine, indodicarbocyanine and indotricarbocyanine skeletons is well described in the prior art. Relevant literature in this respect is, for example: Bioconjugate Chem. 4, 105-111, 1993; Bioconjugate Chem. 7, 356-62, 1996; Bioconjugate Chem. 8, 751-56, 1997; Cytometry 10, 11-19, 1989 and 11, 418-30, 1990; J. Heterocycl. Chem. 33, 1871-6, 1996; J. Org. Chem. 60, 2391-5, 1995; Dyes and Pigments 17, 19-27, 1991, Dyes and Pigments 21, 227-34, 1993; J. Fluoresc. 3, 153-155, 1993; and Anal. Biochem. 217, 197-204, 1994. Additional processes are described in patent publications US 4,981,977; US 5,688,966; US 5,808,044; WO 97/42976; WO 97/42978; WO 98/22146; WO 98/26077; and EP 0 800 831.

Moreover, indotricarbocyanines with altered substituents were synthesized and coupled to biomolecules (described in, i.a., Photochem. Photobiol. 72, 234, 2000; Bioconjugate Chem. 12, 44, 2001; Nature Biotechnol. 19, 237, 2001; J. Biomed. Optics 6, 122, 2001; J. Med. Chem. 45, 2003, 2002). Other examples are found in particular in the publications WO 00/61194 ("Short-Chain Peptide Dye Conjugates as Contrast Agents for Optical Diagnostics"), WO 00/71162, WO 01/52746, WO 01/52743 and WO 01/62156.

The known indotricarbocyanines previously used in the prior art still have drawbacks, however, that impair their efficient use.

Low fluorescence quantum yields of the indotricarbocyanines always exist after a coupling to biomolecules. For the commercially available dye Cy7, Gruber et al. (Bioconjugate Chemn. 11, 696-704, 2000) thus describe that a loss of fluorescence efficiency occurs after coupling to a biomolecule. Becker et al. (Photochem. Photobiol. 72, 234, 2000) describe

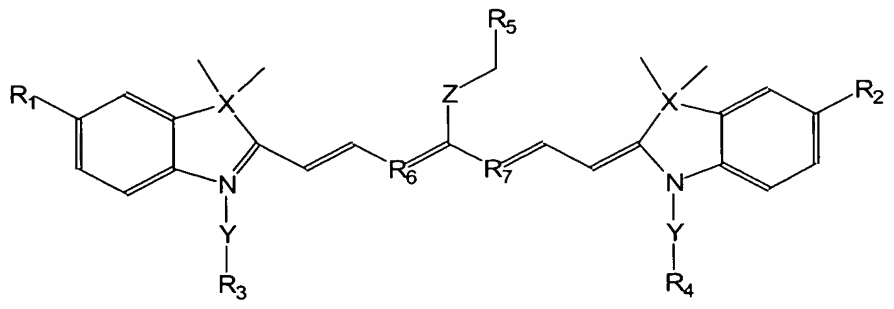
conjugates of an indotricarbocyanine with HSA and transferrin with fluorescence quantum yields of 2.9% or 2.8% and deformed absorption spectra in physiological medium.

In addition, the dye conjugates tend toward aggregation. Becker et al. (Photochem. Photobiol. 72, 234, 2000) for conjugates of an indotricarbocyanine with HSA and transferrin and Licha et al. (Bioconjugate Chem. 12, 44-50, 2001) for receptor-binding peptides describe them as having deformed absorption spectra in physiological medium. These deformations indicate aggregate formation and the fluorescence extinction that occurs as a result. A similar problem exists in the case of an inadequate water solubility of the dyes.

With a reactive group, there is also always inefficient access to derivatives. Gruber et al. (Bioconjugate Chem. 11, 696-704, 2000) thus describe the use of Cy7-bifunctional NHS-esters with two reactive groups, which could potentially produce cross-linking of two bio-molecules. By two carboxy groups in the molecule, however, the synthetic access to derivatives is hampered with only one reactive group and leads to by-products (e.g., contains mono-reactive NHS esters of Cy7 portions of the non-activated and the double-activated Cy7 molecule).

There is thus a continuous need for cyanine dyes that are efficient and easy to produce for the fluorescence diagnosis that reduce or do not have the above-mentioned drawbacks. In addition, these dyes should be well suited for the production of conjugates with biomolecules.

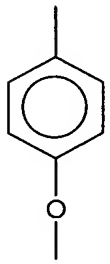
A first object of the invention is achieved by the preparation of an indotricarbocyanine dye of general formula (I),



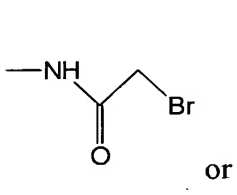
(I)

in which

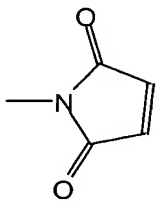
X is O, S or C that is substituted in two places, whereby the substituents can be selected from methyl, ethyl, propyl, isopropyl and/or butyl; Y is CH_2-CH_2 or CH_2-CH_2-CH_2; Z is C_1 to C_5 alkyl, whereby the C atoms are optionally substituted by O or S, or



R_1 to R_4, independently of one another, are SO_3H or H, with the proviso that at least three of R_1 to R_4 are SO_3H, R_5 is -CO-NH-R_8-R_9, -NH-CS-NH-R_8-R_9 or -NH-CO-R_8-R_9, in which R_8 is selected from the group that consists of unbranched C_2-C_{13} alkyl, in which C atoms are optionally replaced by O or S, and R_9 is selected from



or



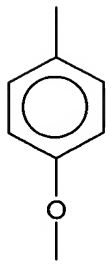
or chloroacetyl, bromoacetyl, iodoacetyl, chloroacetamido, iodoacetamido, chloroalkyl, bromoalkyl, iodoalkyl, pyridyl disulfide and vinyl sulfonamide, and in which R_6 and R_7 are CH or are connected by a C_3-alkyl to a hexyl ring, which optionally can be substituted in para-position with a C_1 to C_4-alkyl radical, and salts and solvates of this compound.

Preferred is an indotricarbocyanine dye of this invention, in which Y is CH_2-CH_2; Z is C_1 to C_5 alkyl, whereby the C atoms are optionally substituted by O or S, and in which R_6 and R_7 are CH, and salts and solvates of this compound.

More preferred is an indotricarbocyanine dye of this invention, in which Z is C_1-C_5 alkyl.

Even more preferred is an indotricarbocyanine dye of this invention, in which

Z is



and R_6 and R_7 are connected to a hexyl ring via C_3-alkyl.

Fluorescence dyes from the class of cyanine dyes, in particular indotricarbocyanines with an absorption and fluorescence maximum in the spectral range of 700 to 900 nm, of a thiol-specific reactive group and three, preferably four, sulfonate groups, are thus subjects of this invention. The latter are used to increase water solubility.

It could now be found, surprisingly enough, that the indotricarbocyanines according to the invention with the above-mentioned structure (compact position of 3-4 sulfonate groups with sulfonatoethyl radicals) have a high fluorescence quantum yield of > 15% and that the fluorescence quantum yield after coupling to biomolecules remains approximately unchanged (maximum loss of about 10%). The absorption spectra of the conjugates show, moreover, no deformation of the dye absorption in the NIR range at about 750 nm. Good hydrophilia, reduced aggregation and increased fluorescence quantum yield relative to conventional indotricarbocyanines or Cy7 derivatives with less than three sulfonate groups, especially in the case of Cy7 and other known structures, are thus produced.

Another essential aspect in the preparation of the cyanine dyes for fluorescence diagnosis according to the invention relates to those derivatives that have reactive functional groups to make possible a covalent coupling to target-specific biomolecules. Suitable derivatives are, e.g., NHS esters and isothiocyanates (Bioconjugate Chem. 4, 105-111, 1993; Bioconjugate Chem. 8, 751-56, 1997), which react with amino groups, such as, for example, maleimides, alpha-haloketones or alpha-haloacetamides (Bioconjugate Chem. 13, 387-391, 2002; Bioconjugate Chem. 11, 161-166, 2000), which react with thiol groups. Other bifunc-

tional linkers can originate from the group that comprises arylenediisothiocyanate, alkylenediisothiocyanate, bis-N-hydroxy-succinimidylesters, hexamethylenediisocyanate and N-(γ-maleimidobutyryloxy)succinimide ester.

WO 01/77229 describes cyanine dyes with a combination of sulfoaryl groups, alkyl substituents in *meso*-position of the methine chain and at least one reactive group that makes possible the binding to biomolecules. The embodiments relate to indodicarbocyanines (polymethine chain that consists of 5 C atoms), however, and the compounds have no reactive group in *meso*-position.

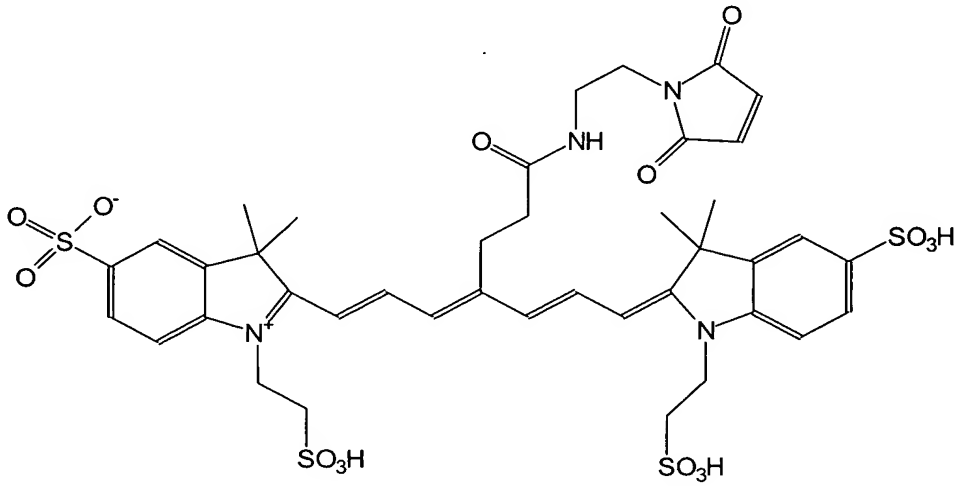
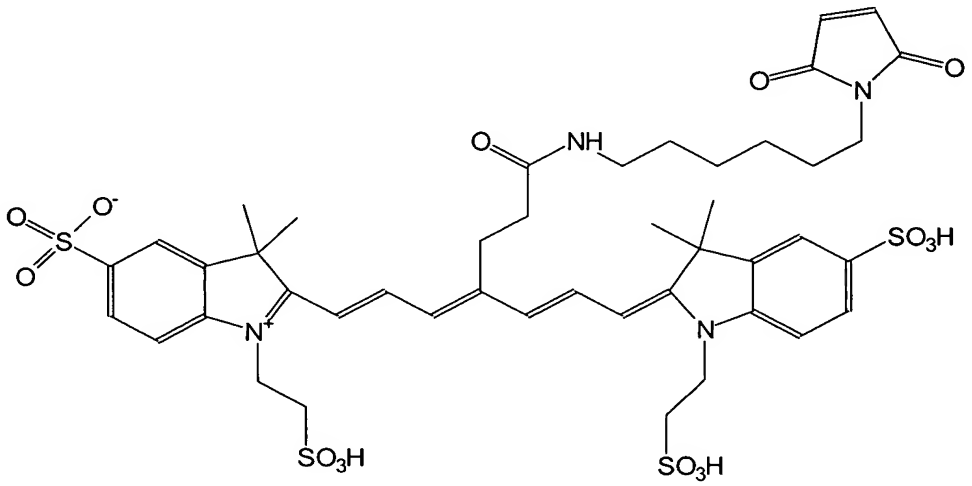
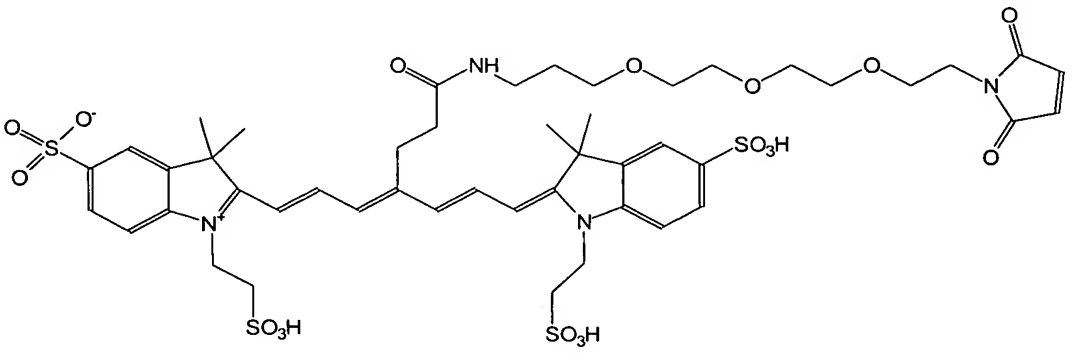
WO 00/16810 ("Near-Infrared Fluorescent Contrast Agent and Fluorescence Imaging") describes indotricarbocyanines, i.a., with substituents in the *meso*-position of the C7-polymethine chain. It is not indicated, however, how reactive groups can be introduced or produced.

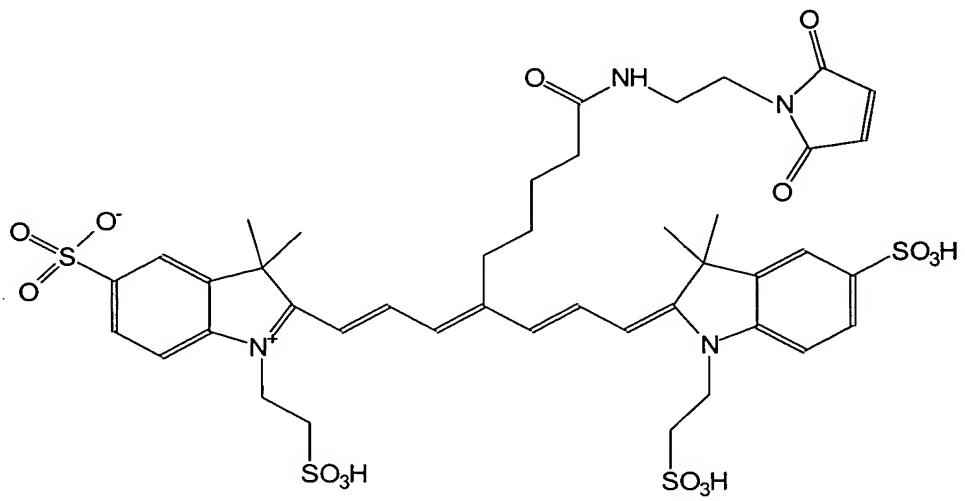
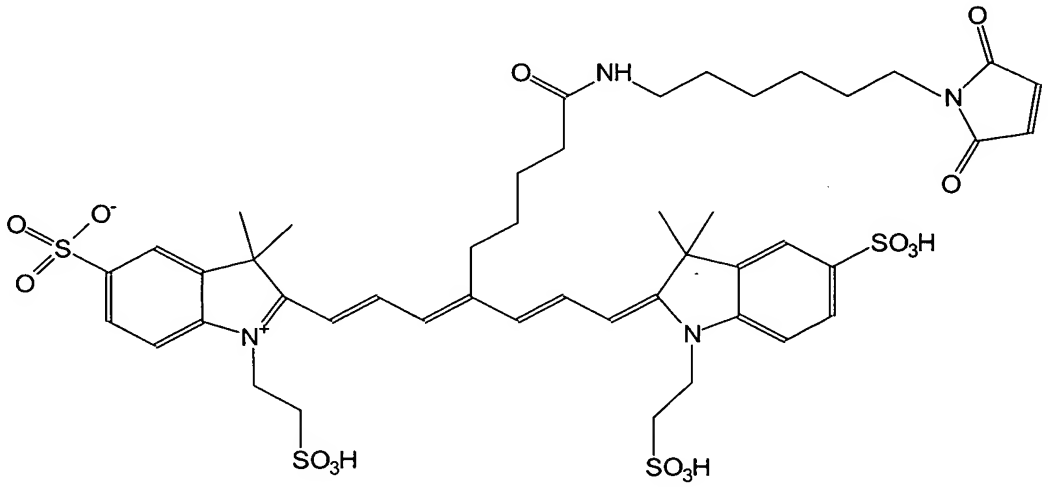
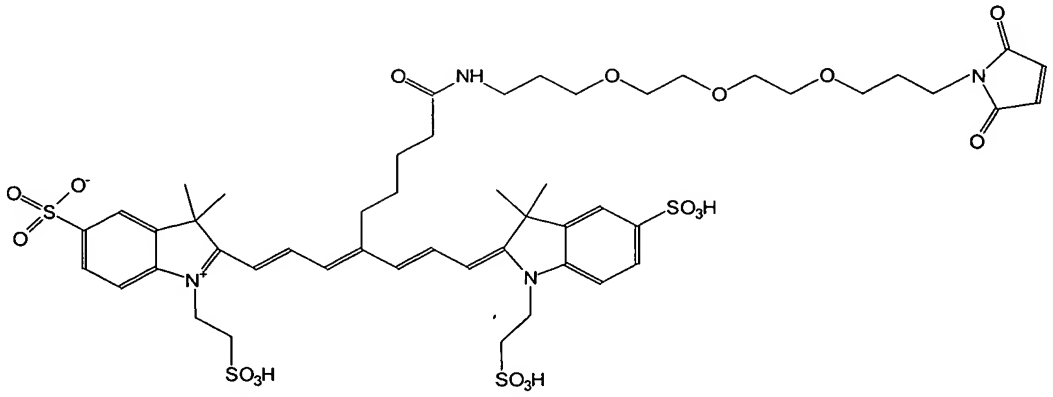
Another aspect of this invention relates to an indotricarbocyanine dye, in which R_5 is COOH or NH_2.

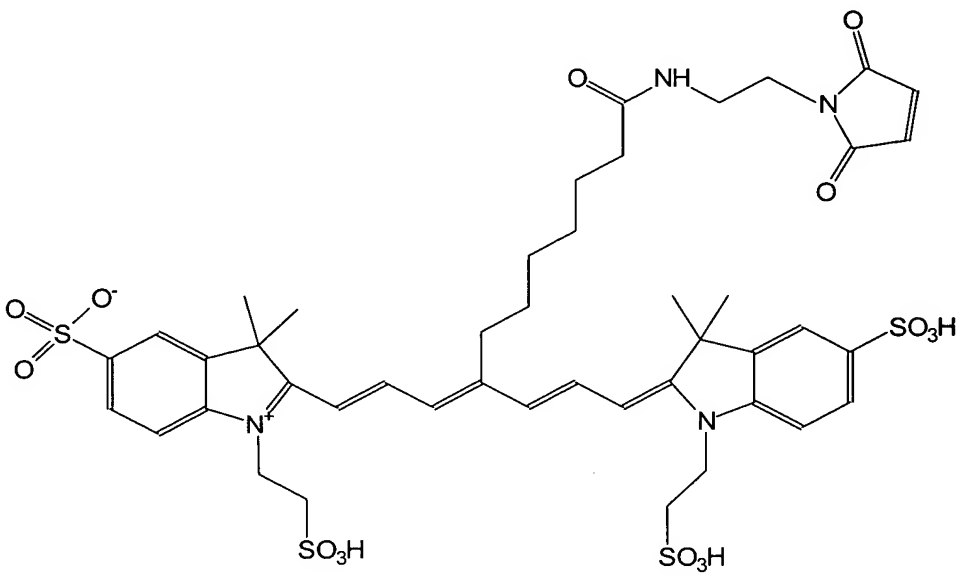
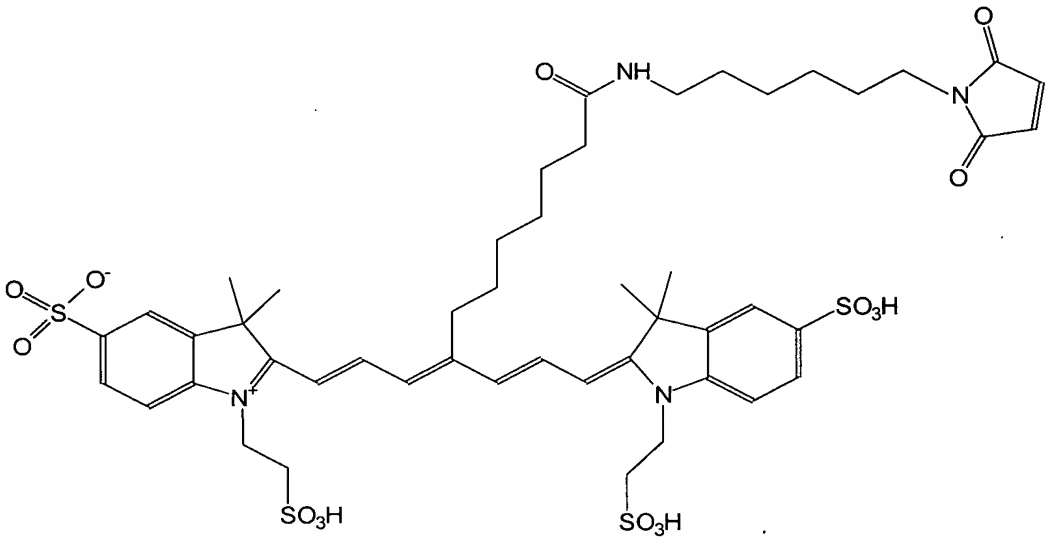
The published derivatives are based primarily on the Cy3-, Cy5-, Cy5.5- and Cy7-basic structure (commercially available from Amersham Pharmacia Biotech; US 5,268,486; Cy3 = indocarbocyanine, Cy5=indodicarbocyanine, Cy7=indotricarbocyanine). Thiol group-reactive derivatives are of special interest, since the latter allow a directed conjugation with biotechnological cysteines that are positioned specifically in biomolecules. The prior art is concentrated here primarily on Cy3 and Cy5 derivatives.

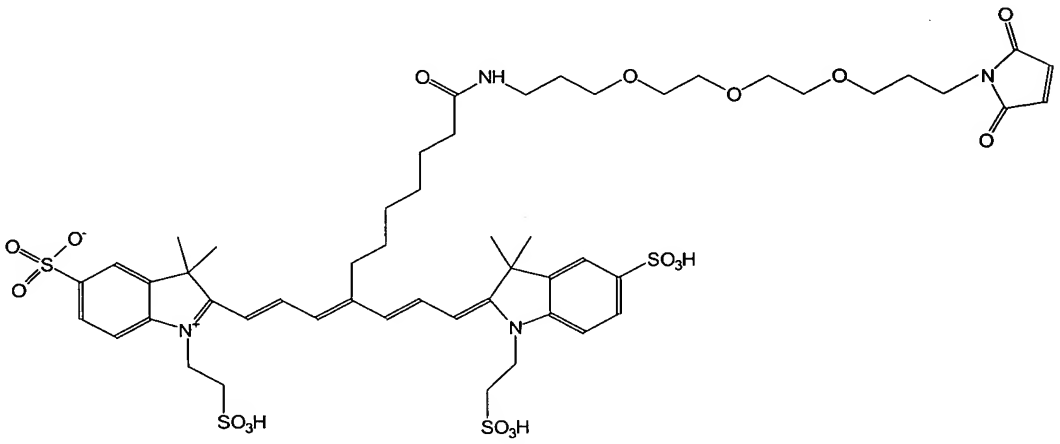
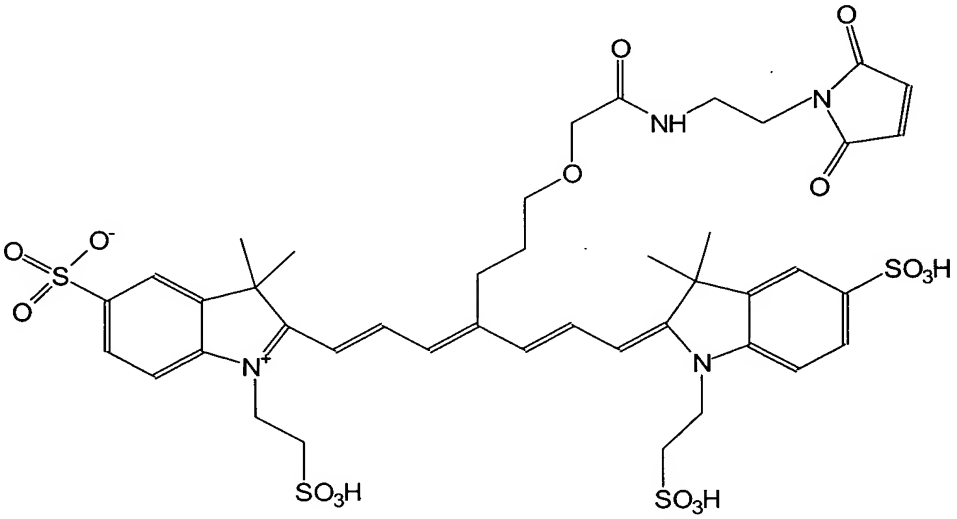
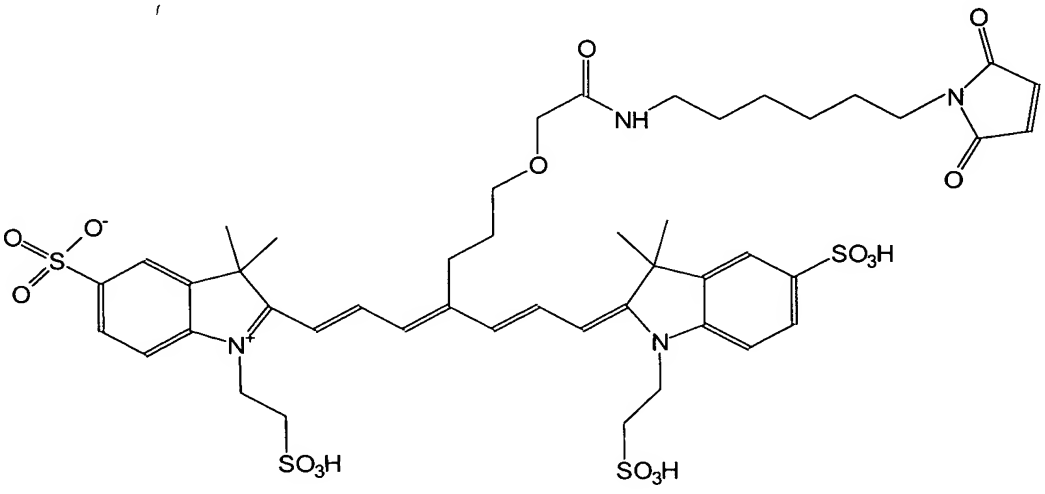
Especially preferred indotricarbocyanine dyes according to the invention are selected from the dyes with formulas (II) to (XX) that are listed in Table 1 below:

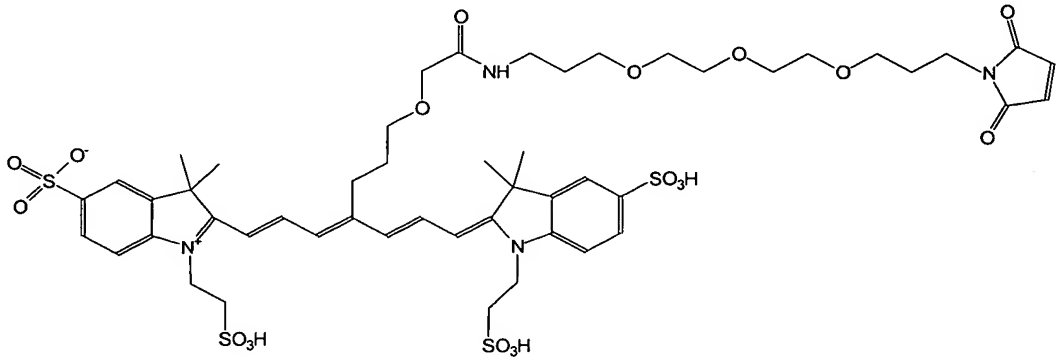
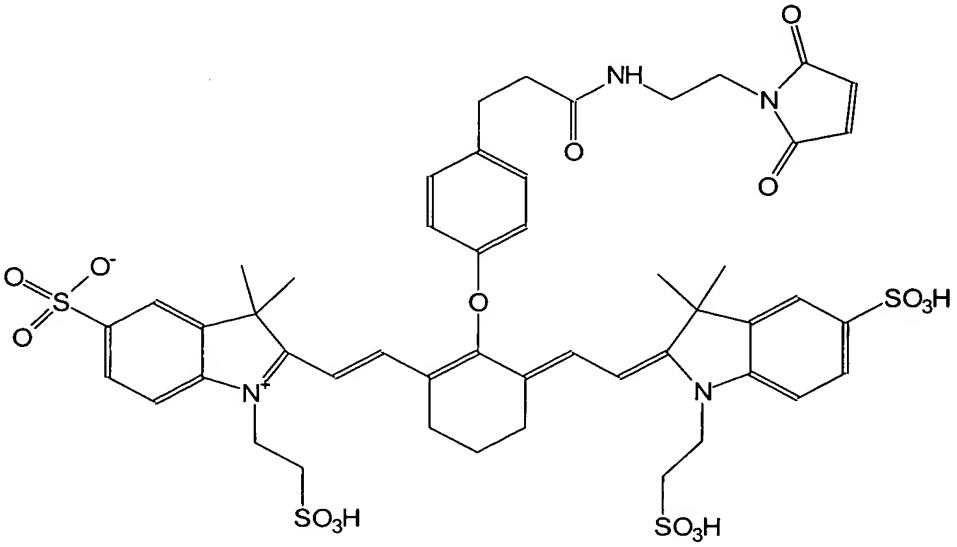
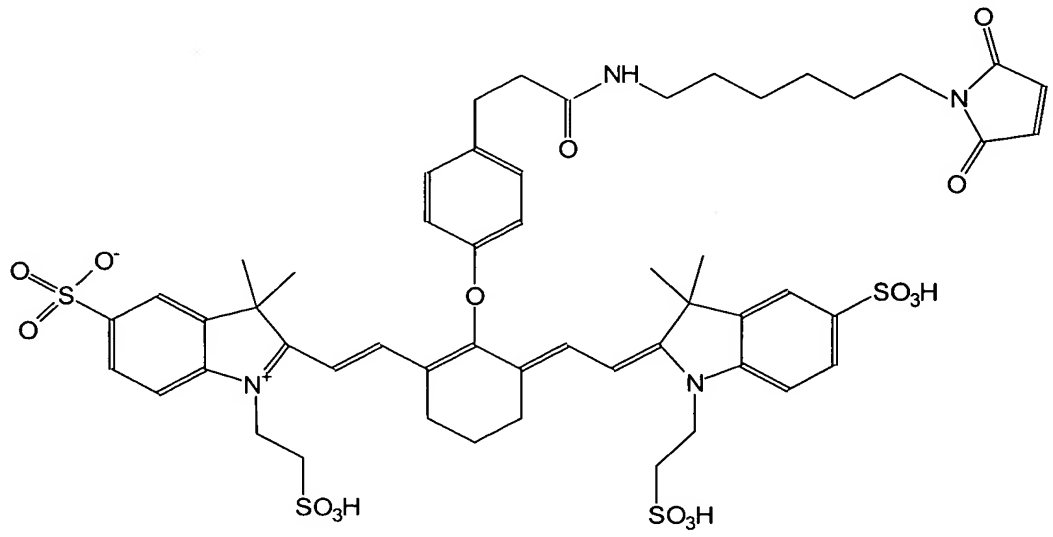
Table 1: Preferred Dyes According to the Invention

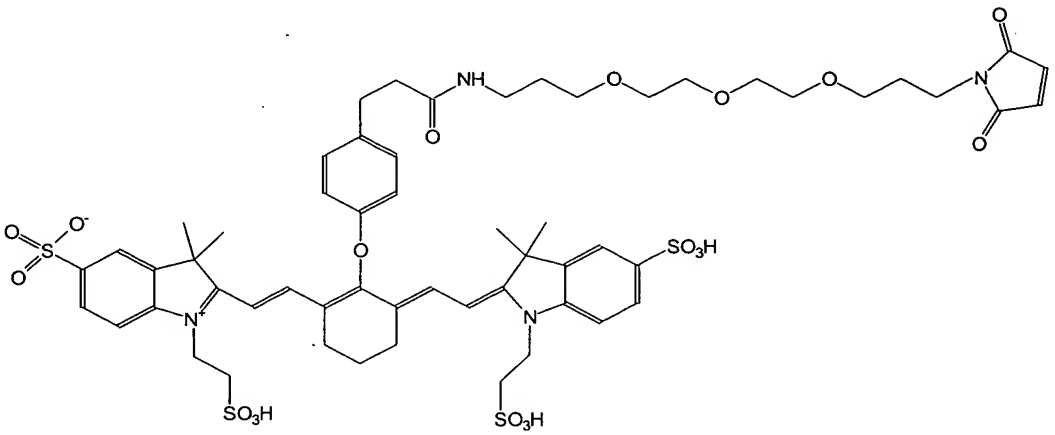
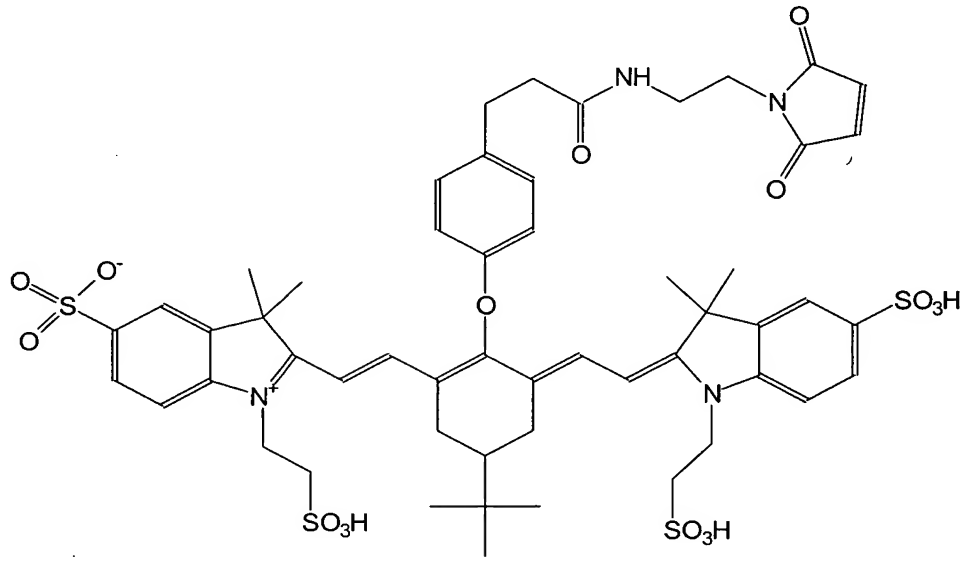
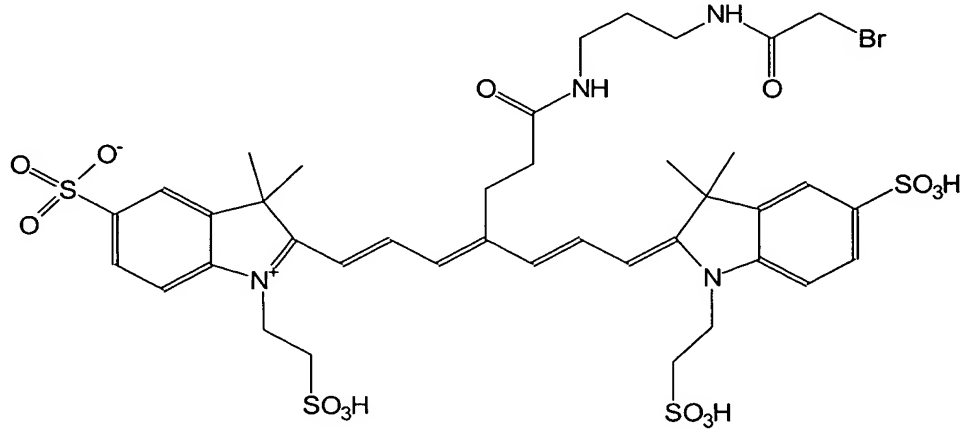
Formula	
(II) Example 1	
(III) Example 2	
(IV) Example 3	

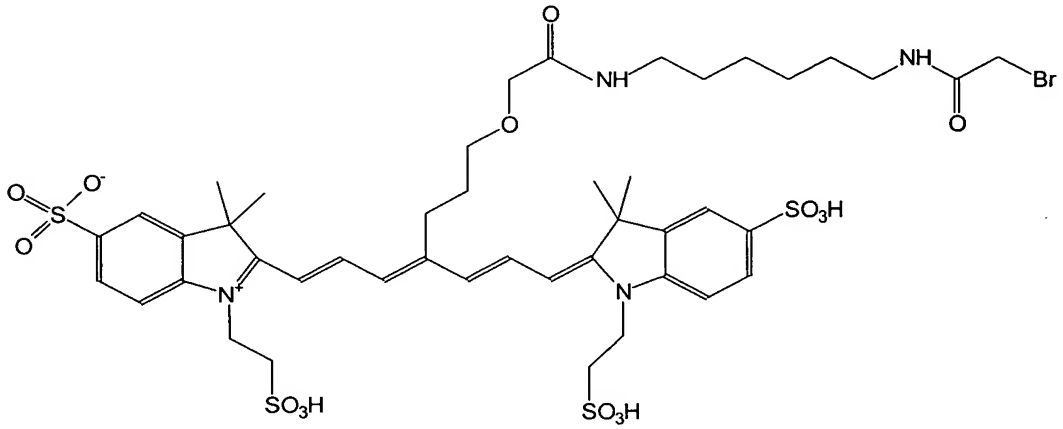
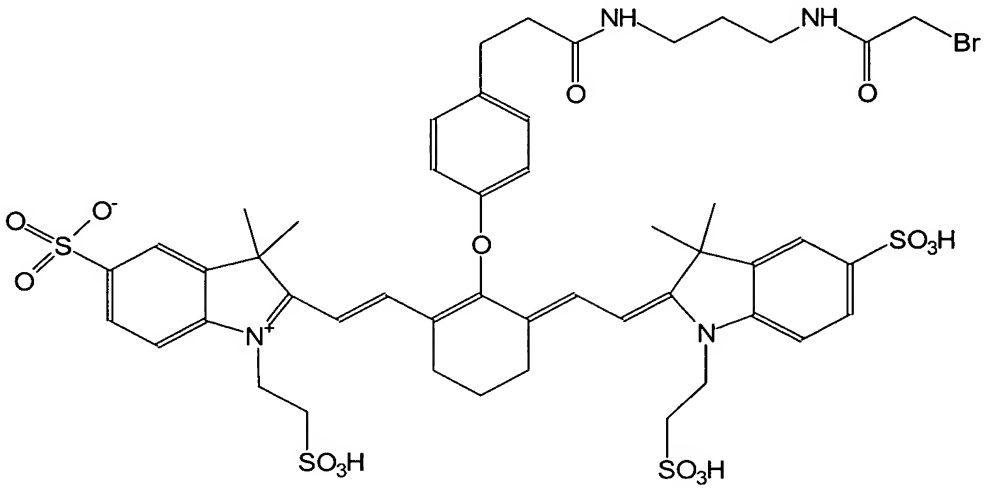
Formula	
(V) Example 4	
(VI) Example 5	
(VII) Example 6	

Formula	
(VIII) Example 7	
(IX) Example 8	

Formula	
(X) Example 9	
(XI) Example 10	
(XII) Example 11	

Formula	
(XIII) Example 12	
(XIV) Example 13	
(XV) Example 14	

Formula	
(XVI) Example 15	
(XVII) Example 16	
(XVIII) Example 18	

Formula	
(XIX) Example 17	
(XX) Example 19	

Another aspect of this invention relates to a process for the production of an indotricarbocyanine dye of this invention. In this case, a simple access via 4-substituted pyridines was found. Surprisingly enough, various 4-substituted pyridines in high yields could be converted by means of the Zincke reaction (Zincke-König reaction, see Römpps Chemie Lexikon [Römpps Chemical Dictionary], 10th Edition, page 5067) in *meso*-substituted glutaconaldehyde-dianilide (precursors to cyanine dye).

In addition to the simple and efficient synthesis of 4-substituted pyridines, the symmetrical structure of the dyes of this invention opens up the possibility of a defined derivatiza-

tion with a thiol-group-selective reactive group in symmetrical *meso*-position of the molecule.

The further derivatization to thiol group-reactive compounds was thus carried out. Thiol group-reactive functionalities are, e.g., maleinimide (maleimide), chloroacetyl, bromoacetyl, iodoacetyl, chloroacetamido, bromoacetamido, iodoacetamido, chloroalkyl, bromoalkyl, iodoalkyl, pyridyl disulfide and vinyl sulfonamide.

Still another aspect of this invention relates to a process for the production of a conjugate that comprises coupling an indotricarbocyanine dye of this invention to a biomolecule. Within the scope of this invention, "biomolecule" is to be defined as any molecule of biological origin that has a biological activity, in particular enzymatic activity or binding of substances of synthetic or biological origin, such as, for example, pharmaceutical agents, peptides, proteins, receptors or nucleic acids. In turn, preferred biomolecules are proteins, such as, for example, enzymes, peptides, antibodies and antibody fragments (such as, e.g., single chain, Fab, $F(ab)_2$, diabodies, etc.), lipoproteins, nucleic acids, such as, for example, oligonucleotides or polynucleotides from DNA or RNA, aptamers, PNA, and sugars, such as, for example, mono-, di-, tri-, oligo- and polysaccharides.

The synthesis and biological characterization of cyanine dye conjugates with biomolecules, such as peptides, antibodies and fragments thereof and proteins for *in-vivo* fluorescence diagnosis of tumors, is described in the prior art in various publications. In this case, primarily the above-mentioned Cy3, Cy5, Cy5.5 and Cy7 were used (see in this respect, i.a., Nature Biotechnol. 15, 1271, 1997; Cancer Detect. Prev. 22, 251, 1998; J. Immunol. Meth. 231, 239, 1999; Nature Biotechnol. 17, 375, 1999; Nature Medicine 7, 743, 2001).

Another aspect of this invention relates to a conjugate of an indotricarbocyanine dye according to the invention with a biomolecule that was produced according to a process of the invention. This conjugate can be characterized in that it comprises a biomolecule as defined above, whereby as a biomolecule, at least one biomolecule, selected from peptides, proteins,

lipoproteins, antibodies or antibody fragments, nucleic acids, such as, for example, oligonucleotides or polynucleotides of DNA or RNA, aptamers, PNA, and sugars, such as, for example, mono-, di-, tri-, oligo- and polysaccharides, is more preferred. The coupled protein can thus be characterized in that it is selected from the group of skeletal proteins or soluble proteins of the body. Quite especially preferred are serum proteins (e.g., HSA), antibodies/antibody fragments, such as, e.g., an scFv-fragment or F(ab), as well as peptides, BSA, egg albumin or a peroxidase derived therefrom.

Thus known from the prior art are, e.g., antibodies that are directed against molecules that are expressed intensively in the angiogenetically active tissue and only to a very low level in the adjoining tissue (see WO 96/01653). Of special interest are antibodies that are against the receptors for vascular growth factors, receptors with endothelial cells to which inflammation mediators bind, and matrix proteins that are expressed specifically in the formation of new vessels. Preferred are other antibodies or antibody fragments that are directed against the matrix protein EDB-fibronectin and conjugates therefrom according to the invention. EDB-fibronectin, also known as oncofetal fibronectin, is a splice variant of the fibronectin, which is formed specifically around newly formed vessels in the process of angiogenesis. Especially preferred are antibodies L19, E8, AP38 and AP39 against the EDB-fibronectin (Cancer Res 1999, 59, 347; J Immunol Meth 1999, 231, 239; Protein Expr Purif 2001, 21, 156).

Preferred is a conjugate according to the invention that is characterized in that the indotricarbocyanine dye is coupled to the biomolecule via an SH group, especially via an SH group to a cysteine. Optionally even more preferably produced therefrom are those antibodies and their fragments that are produced by recombinant techniques, such that on the C-terminus or the N–terminus (within the outside 1-10 amino acids), they contain a cysteine that does not form any intramolecular S-S-bridges and therefore can be used for coupling to the dyes according to the invention.

Another aspect of this invention relates to a diagnostic kit that comprises an indotricarbocyanine dye of this invention and/or a conjugate of this invention. In addition, the kit can contain additional adjuvants for implementing an *in-vivo* diagnosis of, in particular , tumors. These adjuvants are, for example, suitable buffers, vessels, detection reagents or directions for use. The kit preferably contains all materials for an intravenous administration of the dyes according to the invention. Special embodiments of such kits according to the invention are, for example, as follows.

A first vessel that contains the antibody (biomolecule) with a free SH group and standard buffers/additives either as a solution or freeze-dried material. Another vessel that contains the dyes according to the invention as solution (common additives) or freeze-dried material in a molar ratio of 0.1 to 1 (10x deficit in an equimolar quantity). The dye-containing vessel is optionally mixed with buffer or distilled water and added to the biomolecule-containing vessel, incubated for 1-10 minutes and used directly as an injection solution.

In another embodiment, the kit is present in a two-chamber system (e.g., a syringe), which in one chamber contains the antibody solution, and physically separated by a breachable wall in a second chamber contains the dye as solution or solid material. After the wall is broken, a mixture and production of the injection solution is carried out.

A last aspect of this invention then relates to the use of a conjugate according to the invention as a fluorescence contrast medium for *in-vivo* diagnosis of tumors. The absorption maximum of Cy7 in this connection is in the range of 745 nm and is thus especially suitable for the *in-vivo* detection of fluorescence from deeper tissue layers (see above). Cy7 derivatives with thiol group-selective reactive groups are not yet described, however. In addition, the use of antibody conjugates for detection of the edge areas of tumors is already described in WO 01/23005 (Antibody Dye Conjugates for Binding to Target Structures of Angiogenesis in Order to Intraoperatively Depict Tumor Peripheries), but not with use of advantageous dyes according to the invention.

The invention is now to be further described in terms of the following examples and figures without, however, being limited thereto.

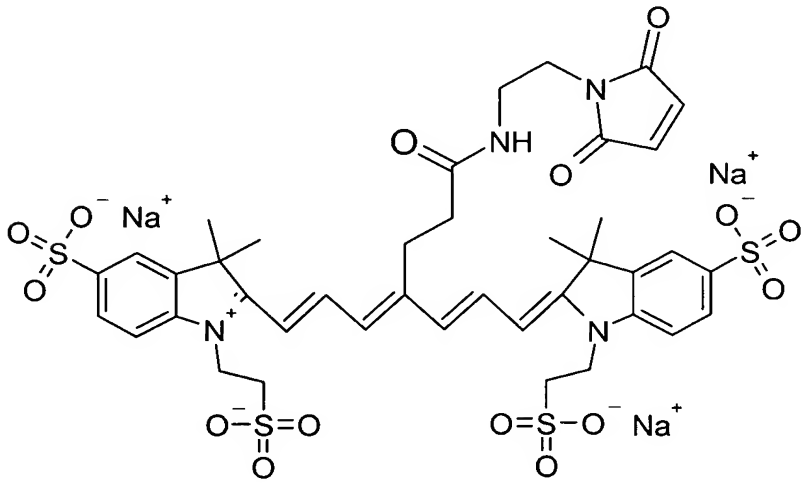
Figure 1 shows the standardized absorption and fluorescence spectrum of conjugate K11 (A) and K15 (B) (see Table 2) in PBS, and

Figure 2 shows the results of the imaging properties of the conjugates according to the invention of Example 24 with: substance: conjugate K15; tumor: F9 teratocarcinoma in the right rear flank of the mouse; dose: 50 nmol/kg of body weight (data relative to the dye); excitation: 740 nm (diode laser); detection: CCD-camera (Hamamatsu) with a 802.5 ± 5 nm bandpass filter and the times: before injection, and 1 hour, 6 hours and 24 hours after injection. The position of the tumor is identified by arrows.

Examples

Examples 1 –16: Synthesis of Indotricarbocyanine Dyes with Maleimide Groups

Example 1: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}-ethyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula II)



a) 1-(2-Sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid, internal salt

10 g (0.04 mol) of 2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (*Bioconjugate Chem 1993, 4, 105*), 6.8 g (0.04 mol) of 2-chloroethanesulfonic acid chloride and 4.2 g (0.04 mol) of triethylamine are refluxed in 200 ml of acetonitrile for 6 hours. The precipitate is suctioned off and dried. Yield 5.0 g (35% of theory). *Anal Biochem 1994, 217, 197*

b) 3-Pyridin-4-yl-propionic acid-*tert*-butyl ester

20 g (89 mmol) of t-butyl-P,P-dimethylphosphonoacetate in 50 ml of THF is added in drops at 0°C to a suspension of 3.9 g (98 mmol) of sodium hydride (60 % in mineral oil) in

250 ml of THF. After 1 hour of stirring at 0°C, a solution of 10 g (93 mmol) of pyridine-4-carbaldehyde in 50 ml of tetrahydrofuran is added in drops, and the reaction mixture is stirred for 1 hour at 0°C and for 18 hours at room temperature. The precipitated solid is removed by filtration, and the solution is concentrated by evaporation. The residue is dissolved in isopropanol while being heated, non-soluble portions are filtered off, and the solution is cooled to 0°C for crystallization. The solid that is produced is filtered off, stirred with hexane, filtered and dried. The intermediate product (15.3 g) is hydrogenated in 150 ml of ethanol with 0.15 g of 10% palladium/activated carbon for 6 hours. The catalyst is filtered off, the solution is concentrated by evaporation, and the residue is filtered on silica gel (mobile solvent diethyl ether). 13.0 g of a light yellow oil (71% of theory) is obtained.

c) 3-[2-(*tert*-Butyloxycarbonyl)ethyl]glutaconaldehyde-dianilide-hydrobromide

A solution of 10 g (48 mmol) of 3-pyridin-4-yl-propionic acid-*tert*-butyl ester in 150 ml of diethyl ether is mixed with 8.9 g (96 mmol) of aniline and then mixed at 0°C with a solution of 5.4 g (48 mmol) of bromocyanogen in 2 ml of diethyl ether. After 3 hours of stirring at 0°C, the red solid that is produced is filtered off, washed with ether and vacuum-dried.

Yield: 20.3 g (92% of theory)

d) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(2-carboxyethyl) hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

A suspension of 1.0 g (2.2 mmol) of 3-[2-(*tert*-butyloxycarbonyl)ethyl]-glutaconaldehyde-dianilide-hydrobromide (Example 1c)) and 1.5 g (4.4 mmol) of 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (Example 1a)) in 20 ml of acetic acid anhydride and 5 ml of acetic acid is mixed with 0.75 g (9.1 mmol) of sodium acetate and stirred for 1 hour at 120°C. After cooling, it is mixed with diethyl ether, the precipitated

solid is filtered off and purified by chromatography (RP-C18-silica gel, mobile solvent water/methanol) and the product is freeze-dried (0.5 g). The cleavage of the protective group is carried out by stirring the intermediate product in 4 ml of dichloromethane/1 ml of trifluoroacetic acid for 1 hour. After concentration by evaporation and chromatographic purification (RP-C18-silica gel, mobile solvent water/methanol), 0.45 g (23% of theory) of a blue lyophilizate is obtained.

e) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}ethyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

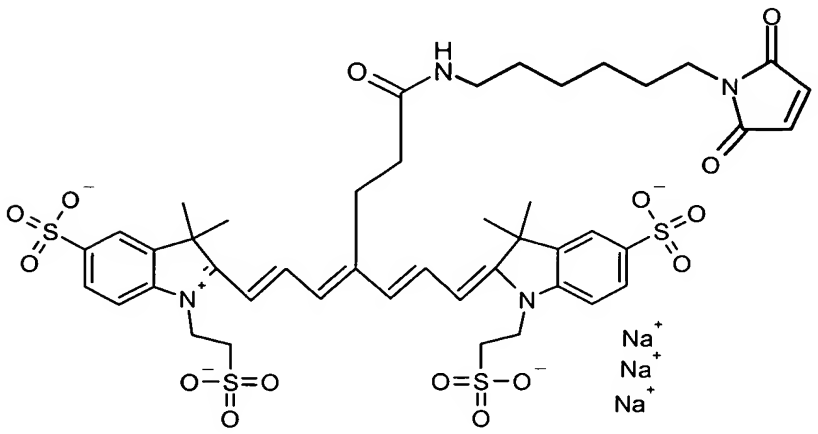
0.4 g (0.45 mmol) of the title compound of Example 1d) and 45 mg (0.45 mmol) of triethylamine are dissolved in 10 ml of dimethylformamide, mixed at 0°C with 0.15 g (0.45 mmol) of TBTU and stirred for 10 minutes. Then, a solution of 0.17 g (0.68 mmol) of N-(2-aminoethyl)maleimide-trifluoroacetate (*Int J Pept Protein Res 1992, 40, 445*) and 68 mg (0.68 mmol) of triethylamine in 0.5 ml of dimethylformamide is added, and it is stirred for 1 hour at room temperature. After 10 ml of diethyl ether is added, the solid is centrifuged off, dried and purified by means of chromatography (RP C-18 silica gel, gradient methanol/water).

Yield: 0.30 g of a blue lyophilizate (65% of theory).

Elementary analysis: Cld.: C 47.24 H 4.26 N 5.51 S 12.61 Na 6.78

Fnd.: C 47.74 H 4.47 N 5.40 S 11.99 Na 7.02

Example 2: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(2-{[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl]carbamoyl}ethyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula III)

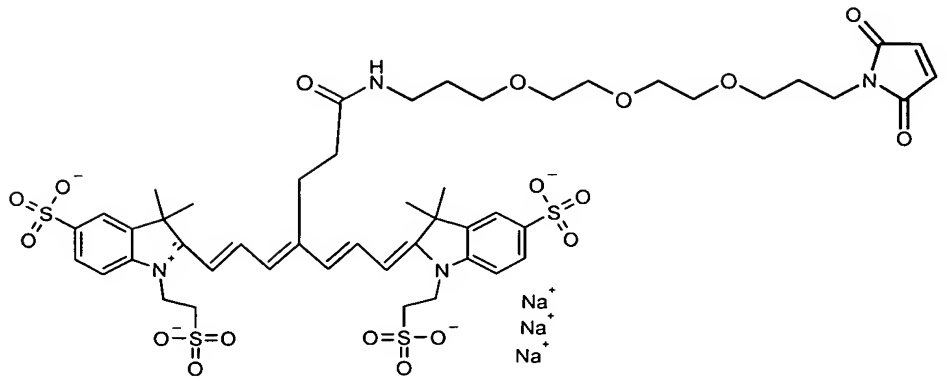


The synthesis is carried out analogously to Example 1e) from 0.4 g (0.45 mmol) of the title compound of Example 1d) and 0.21 g (0.68 mmol) of N-(6-aminohexyl)maleimide-trifluoroacetate (*Int J Pept Protein Res 1992, 40, 445*). Yield: 0.38 g of a blue lyophilizate (81% of theory).

Elementary analysis: Cld.: C 49.25 H 4.79 N 5.22 S 11.95 Na 6.43

Fnd.: C 48.96 H 4.92 N 5.32 S 11.88 Na 6.56

Example 3: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(2-{[13-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4,7,10-trioxatridecyl]carbamoyl}ethyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula IV)

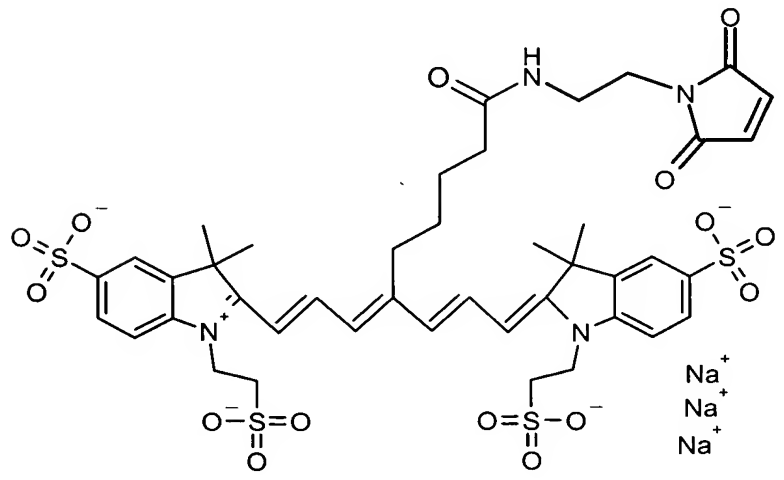


The synthesis is carried out analogously to Example 1e) from 0.4 g (0.45 mmol) of the title compound of Example 1d) and 0.28 g (0.68 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoroacetate (*Int J Pept Protein Res 1992, 40, 445*). Yield: 0.27 g of a blue lyophilizate (51% of theory).

Elementary analysis: Cld.: C 48.97 H 5.05 N 4.76 S 10.89 Na 5.86

Fnd.: C 49.22 H 5.16 N 4.62 S 10.67 Na 5.66

Example 4: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(4-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}-butyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula V)



a) (3-*tert*-Butoxycarbonyl-propyl)-triphenyl-phosphonium bromide

50 g (0.30 mol) of 4-bromobutyric acid is mixed drop by drop in 400 ml of THF at -40°C with 187 g (0.89 mol) of trifluoroacetic acid anhydride. After 30 minutes of stirring at –40°C, 400 ml of tert-butanol/30 ml of THF is added in drops within 1 hour. After 16 hours of stirring at room temperature, the reaction mixture is poured onto an ice-cooled sodium carbonate solution, the aqueous phase is extracted three times with diethyl ether, and the organic phases are dried on sodium sulfate and concentrated by evaporation. The residue is distilled in a vacuum (boiling point 72°C/0.9 mbar; yield: 41 g). The reaction to form phosphonium salt is carried out by reflux-heating 41 g (0.18 mol) of intermediate product, 44.6 g (0.17 mol) of triphenylphosphine and 32.5 g (0.36 mol) of sodium bicarbonate in 250 ml of acetonitrile for 20 hours. The reaction mixture is filtered, concentrated by evaporation, and the residue is brought to crystallization by stirring with diethyl ether. Yield: 58.5 g (40% of theory, relative to 4-bromobutyric acid) of a white solid.

b) 5-Pyridin-4-yl-pentanoic acid-t-butyl ester

A solution of 14 g (28 mmol) of (3-*tert*-butoxycarbonyl-propyl)-triphenyl-phosphonium bromide (Example 4a)) in 100 ml of anhydrous THF is mixed at –40°C in an air-free environment within 20 minutes with 17.5 ml (28 mmol) of butyllithium (1.6 M in hexane) and stirred for 1 hour at –40°C. A solution of 2.78 g (26 mmol) of 4-pyridinecarbaldehyde in 20 ml of THF is added in drops and stirred for 16 hours at room temperature, then poured onto ice water, the aqueous phase is extracted three times with diethyl ether, and the organic phases are dried on sodium sulfate and concentrated by evaporation. After chromatographic purification (silica gel, mobile solvent hexane/ethyl acetate), the product is obtained as an E,Z-mixture (4:1 after ^{1}H-NMR; 5.0 g). To hydrogenate the double bond, the intermediate product is dissolved in 200 ml of methanol and stirred with 100 mg of

PtO_2 catalyst at room temperature over hydrogen. After filtration and concentration by evaporation, a yellow oil is obtained. Yield: 4.9 g (74% of theory).

c) 3-[4-(*tert*-Butyloxycarbonyl)butyl]glutaconaldehyde-dianilide-hydrobromide

A solution of 4.0 g (17 mmol) of 5-pyridin-4-yl-pentanoic acid-t-butylester in 35 ml of diethyl ether is mixed with 3.2 g (34 mmol) of aniline and then at 0°C with a solution of 1.9 g (17 mmol) of bromocyanogen in 8 ml of diethyl ether. After 3 hours of stirring at 0°C, the red solid that is produced is filtered off, washed with ether and vacuum-dried. Yield: 7.8 g (95% of theory).

d) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(4-carboxybutyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1d) from the title compound of Example 4c) (2.5 mmol) and 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (5 mmol). Yield: 0.85 g (37% of theory) of a blue lyophilizate.

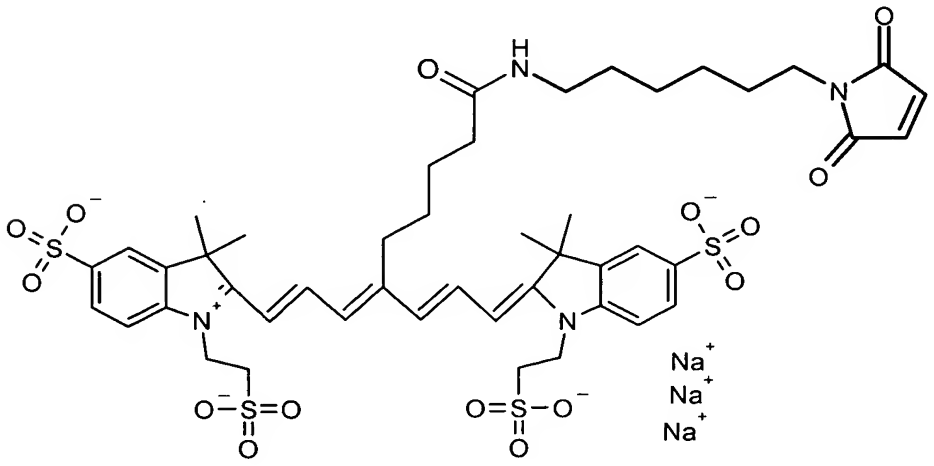
e) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(4-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}-butyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1e) from 0.4 g (0.43 mmol) of the title compound of Example 4d). Yield: 0.31 g (69% of theory) of a blue lyophilizate.

Elementary analysis: Cld.: C 48.27 H 4.53 N 5.36 S 12.27 Na 6.60

Fnd.: C 48.01 H 4.44 N 5.56 S 12.10 Na 6.81

Example 5: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(4-{[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl]carbamoyl}butyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula VI)

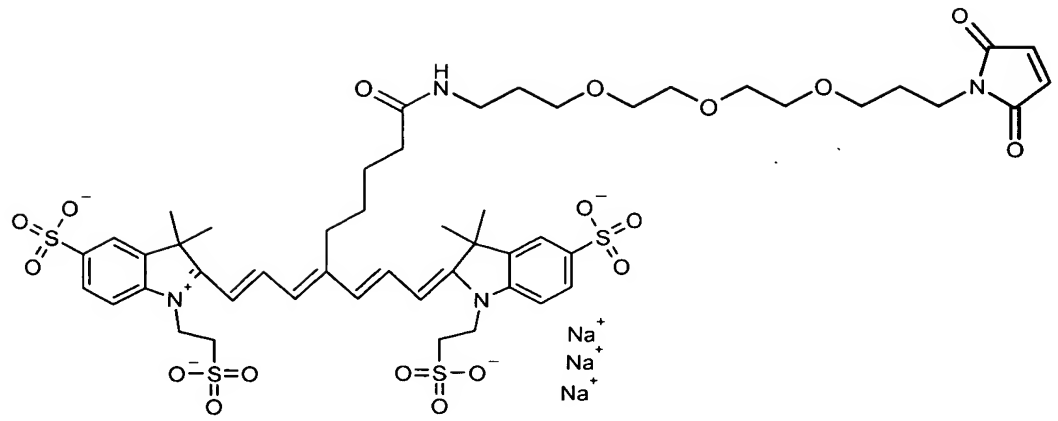


The synthesis is carried out analogously to Example 1e) from 0.4 g (0.43 mmol) of the title compound of Example 4d) and 0.20 g (0.66 mmol) of N-(6-aminohexyl)maleimide-trifluoroacetate. Yield: 0.35 g of a blue lyophilizate (74% of theory).

Elementary analysis: Cld.: C 50.17 H 5.03 N 5.09 S 11.65 Na 6.26

Fnd.: C 49.83 H 4.89 N 5.34 S 12.05 Na 6.42

Example 6: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(4-{[13-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4,7,10-trioxatridecyl]carbamoyl}butyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula VII)

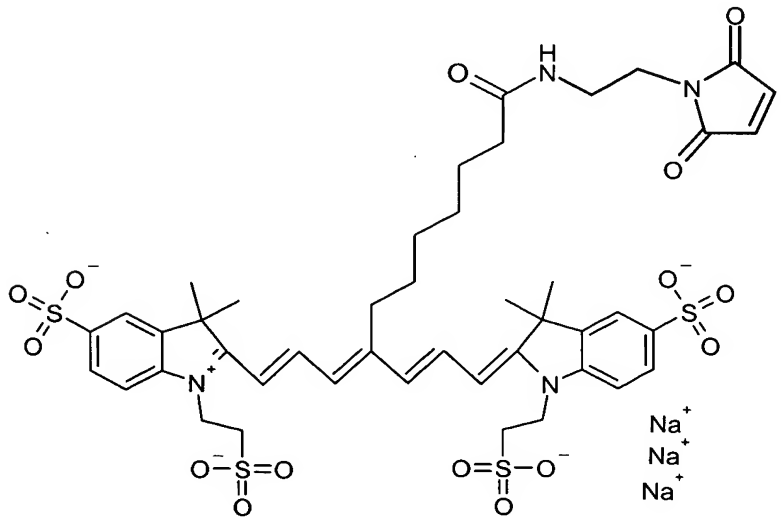


The synthesis is carried out analogously to Example 1e) from 0.4 g (0.43 mmol) of the title compound of Example 1d) and 0.30 g (0.72 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoracetate. Yield: 0.27 g of a blue lyophilizate (52% of theory).

Elementary analysis: Cld.: C 49.83 H 5.27 N 4.65 S 10.64 Na 5.72

Fnd.: C 49.45 H 5.19 N 4.66 S 10.85 Na 5.80

Example 7: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}hexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula VIII)



a) (3-*tert*-Butoxycarbonyl-pentyl)-triphenyl-phosphonium bromide

The production is carried out as described in Example 4a), whereby the intermediate product 6-bromohexanoic acid-*tert*-butyl ester is reacted as a crude product. 79 g of product (69% of theory) is obtained as a viscous, colorless oil from 50 g of 6-bromohexanoic acid.

b) 7-Pyridin-4-yl-heptanoic acid-t-butyl ester

The production is carried out as described in Example 4b). 7.5 g of 7-pyridin-4-yl-heptanoic acid-t-butyl ester (65% of theory) is obtained as a yellow oil from 25 g (48.7 mmol) of (3-*tert*-butoxycarbonyl-pentyl)-triphenyl-phosphonium bromide (Example 7a).

c) 3-[6-(*tert*-Butyloxycarbonyl)hexyl]glutaconaldehyde-dianilide-hydrobromide

A solution of 5.0 g (19 mmol) of 7-pyridin-4-yl-heptanoic acid-t-butyl ester in 30 ml of diethyl ether is mixed with 3.6 g (38 mmol) of aniline and then at 0°C with a solution of 2.1 g (19 mmol) of bromocyanogen in 5 ml of diethyl ether. After 2.5 hours of stirring at 0°C, the red solid that is produced is filtered off, washed with ether and vacuum-dried. Yield: 8.9 g (91% of theory).

d) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-carboxyhexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1d) from the title compound of Example 7c) (3 mmol) and 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (6 mmol). Yield: 1.5 g (54% of theory) of a blue lyophilizate.

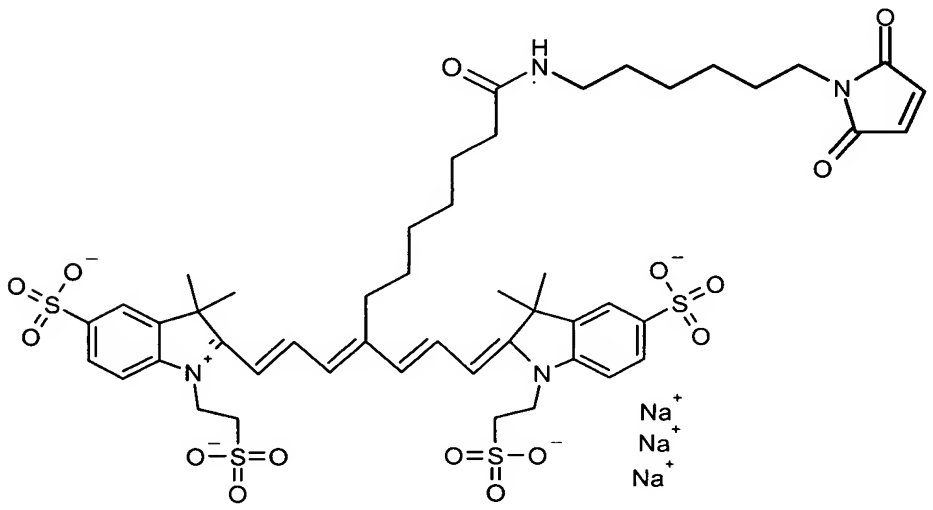
e) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}hexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1e) from 0.4 g (0.43 mmol) of the title compound of Example 7d). Yield: 0.31 g (69% of theory) of a blue lyophilizate.

Elementary analysis: Cld.: C 49.25 H 4.79 N 5.22 S 11.95 Na 6.43

Fnd.: C 48.98 H 4.86 N 5.12 S 11.76 Na 6.77

Example 8: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-{[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl]carbamoyl}hexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula IX)

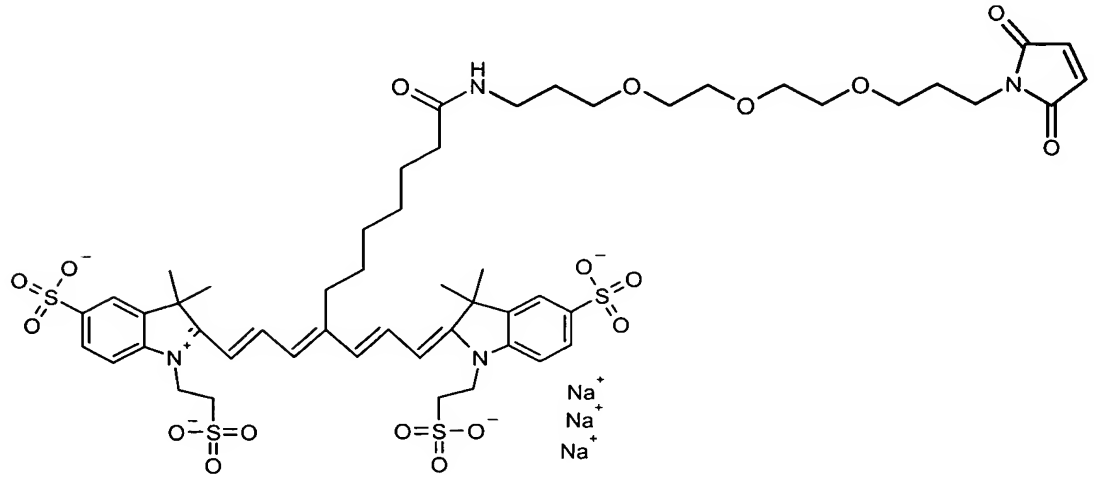


The synthesis is carried out analogously to Example 1e) from 0.5 g (0.53 mmol) of the title compound of Example 7d) and 0.23 g (0.75 mmol) of N-(6-aminohexyl)maleimide-trifluoroacetate. Yield: 0.42 g of a blue lyophilizate (70% of theory).

Elementary analysis: Cld.: C 51.05 H 5.27 N 4.96 S 11.36 Na 6.11

Fnd.: C 50.74 H 5.55 N 4.76 S 11.38 Na 6.35

Example 9: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-{[13-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4,7,10-trioxatridecyl]carbamoyl}hexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula X)

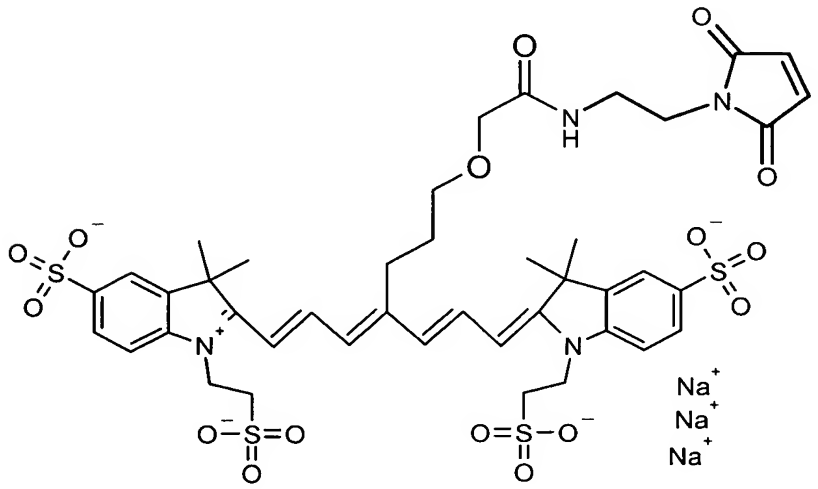


The synthesis is carried out analogously to Example 1e) from 0.5 g (0.53 mmol) of the title compound of Example 7d) and 0.44 g (1.06 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoroacetate. Yield: 0.24 g of a blue lyophilizate (37% of theory).

Elementary analysis: Cld.: C 50.64 H 5.48 N 4.54 S 10.40 Na 5.59

Fnd.: C 50.30 H 5.56 N 4.34 S 10.15 Na 5.73

Example 10: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}-3-oxa-pentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XI)



a) 3-Oxa-6-(4-Pyridinyl)hexanoic acid-*tert*-butyl ester

A solution of 75 g (0.4 mol) of 3-(4-pyridinyl)-1-propanol in 400 ml of toluene/50 ml of THF is mixed with 10 g of tetrabutylammonium sulfate and 350 ml of 32% sodium hydroxide solution. Then, 123 g (0.68 mol) of bromoacetic acid-*tert*-butyl ester is added in drops and stirred for 18 hours at room temperature. The organic phase is separated, and the aqueous phase is extracted three times with diethyl ether. The combined organic phases are washed with NaCl solution, dried on sodium sulfate and concentrated by evaporation. After chromatographic purification (silica gel: mobile solvent hexane:ethyl acetate), 56 g of product (41% of theory) is obtained as a brownish oil.

b) 3-[4-Oxa-5-(*tert*-butyloxycarbonyl)pentyl]glutaconaldehyde-dianilide-hydrobromide

A solution of 5.0 g (20 mmol) of 3-oxa-6-(4-pyridinyl)hexanoic acid-*tert*-butyl ester in 60 ml of diethyl ether is mixed with 3.7 g (40 mmol) of aniline and then at 0°C with a solu-

tion of 2.2 g (20 mmol) of bromocyanogen in 8 ml of diethyl ether. After 1 hour of stirring at 0°C, 50 ml of diethyl ether is mixed, and the red solid that is produced is filtered off, washed with ether and vacuum-dried. Yield: 8.5 g (85% of theory) of a violet solid.

c) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(6-carboxy-4-oxahexyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

A suspension of 3.0 g (6 mmol) of 3-[2-(*tert*-butyloxycarbonyl)ethyl]-glutaconaldehyde-dianilide-hydrobromide (Example 10b)) and 4.2 g (12 mmol) of 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (Example 1a)) in 50 ml of acetic acid anhydride and 10 ml of acetic acid is mixed with 2.5 g (30 mmol) of sodium acetate and stirred for 50 minutes at 120°C. After cooling, it is mixed with diethyl ether, the precipitated solid is filtered off, absorptively precipitated in acetone and dried under high vacuum. After chromatographic purification (RP-C18-silica gel, mobile solvent water/methanol), removal of the methanol in a vacuum and freeze-drying, the title compound is immediately obtained. Yield: 2.3 g (41% of theory) of a blue lyophilizate.

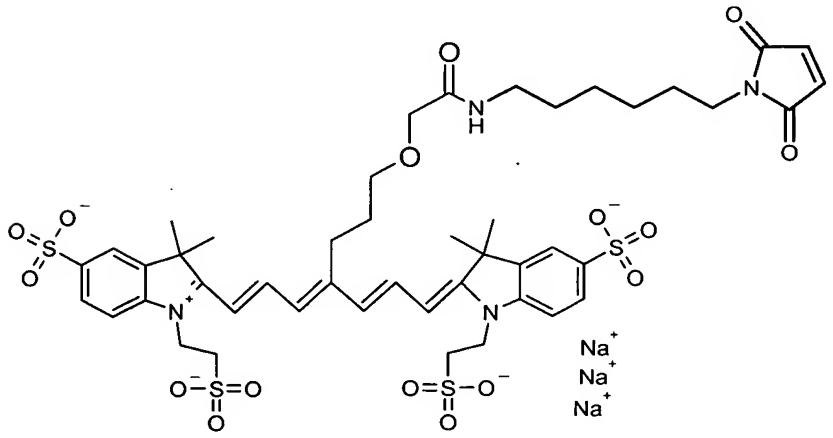
d) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}-3-oxa-pentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1c) from 1.0 g (1.1 mmol) of the title compound of Example 10c). Yield: 0.85 g (73% of theory) of a blue lyophilizate.

Elementary analysis: Cld.: C 47.54 H 4.46 N 5.28 S 12.09 Na 6.50

Fnd.: C 47.97 H 4.65 N 5.10 S 12.02 Na 6.68

Example 11: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl]carbamoyl}-3-oxa-pentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XII)

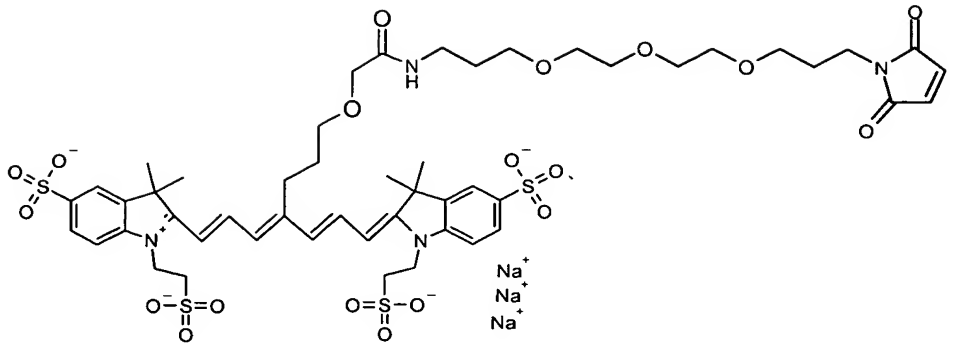


The synthesis is carried out analogously to Example 1e) from 0.5 g (0.55 mmol) of the title compound of Example 10c) and 0.23 g (0.75 mmol) of N-(6-aminohexyl)maleimide-trifluoroacetate. Yield: 0.42 g of a blue lyophilizate (68% of theory).

Elementary analysis: Cld.: C 49.46 H 4.96 N 5.01 S 11.48 Na 6.17

Fnd.: C 48.95 H 5.21 N 5.22 S 11.23 Na 6.60

Example 12: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[13-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4,7,10-trioxatridecyl]carbamoyl}-4-oxapentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XIII)

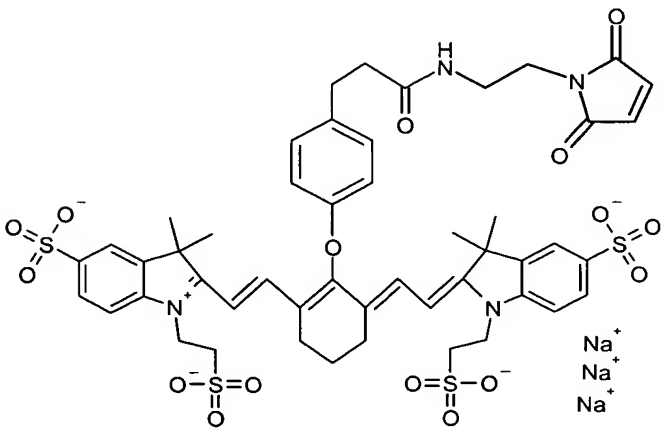


The synthesis is carried out analogously to Example 1e) from 0.5 g (0.55 mmol) of the title compound of Example 10c) and 0.46 g (1.06 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoroacetate. Yield: 0.34 g of a blue lyophilizate (56% of theory).

Elementary analysis: Cld.: C 49.17 H 5.20 N 4.59 S 10.50 Na 5.65

Fnd.: C 49.34 H 5.32 N 4.45 S 10.28 Na 5.56

Example 13: Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1- yl)ethyl]carbamoyl}ethyl)-phenoxy]cyclohex-1-en-3-yliden)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XIV)



a) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-chloro-cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

5.0 g (14.4 mmol) of 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (Example 1a)) and 2.6 g (7.2 mmol) of N-[(3-(anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]aniline hydrochloride (*Aldrich Company*) are refluxed together with 2.5 g (30 mmol) of anhydrous sodium acetate in 100 ml of methanol for 1 hour, cooled, mixed with 150 ml of diethyl ether and stirred overnight. The precipitate is suctioned off, dried and purified by chromatography (silica gel, gradient: dichloromethane/methanol). Yield: 3.8 g (58% of theory) of a blue solid.

b) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-carboxyethyl)phenoxy]cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

0.37 g (2.2 mmol) of 3-(4-hydroxyphenyl)propionic acid in 30 ml of dimethylformamide is mixed with 0.18 g (4.5 mmol) of sodium hydride (60% mineral oil dispersion). After 30 minutes of stirring at room temperature, it is cooled to 0°C, a solution of 2.0 g (2.2 mmol) of the title compound of Example 12a) in 100 ml of dimethylformamide is added in drops and stirred for 2 hours at room temperature. The mixture is quenched with dry ice, and the solvent is removed in a vacuum. The residue is dissolved in methanol, stirred with 200 ml of ether, and the precipitated solid is filtered off. A chromatographic purification is carried out (silica gel, gradient: ethyl acetate/methanol). Yield: 1.9 g of a blue solid (83% of theory).

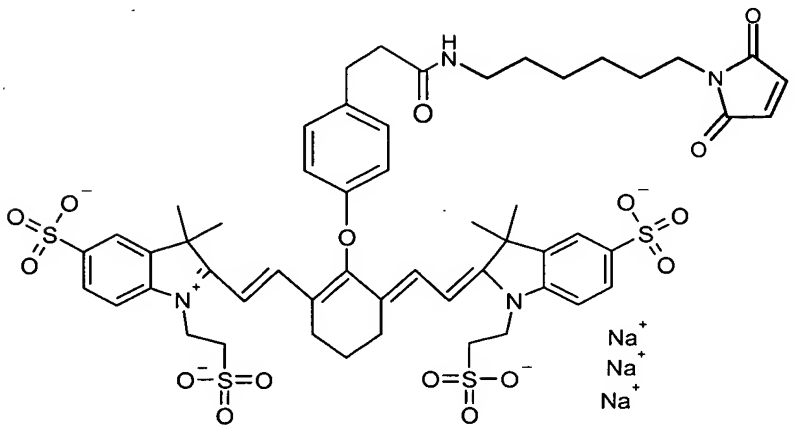
c) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}ethyl)-phenoxy]cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

0.1 mg (0.10 mmol) of the title compound of Example 12b) is reacted as described in Example 1e) with TBTU and N-(2-aminoethyl)maleimide-trifluoroacetate in the presence of triethylamine, and the product that is obtained is purified by chromatography. Yield: 93 mg of a blue lyophilizate (81% of theory).

Elementary analysis: Cld.: C 51.21 H 4.47 N 4.88 S 11.16 Na 6.00

Fnd.: C 51.50 H 4.55 N 4.95 S 10.93 Na 6.15

Example 14: Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1- yl)hexyl]carbamoyl}ethyl)-phenoxy]cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XV)

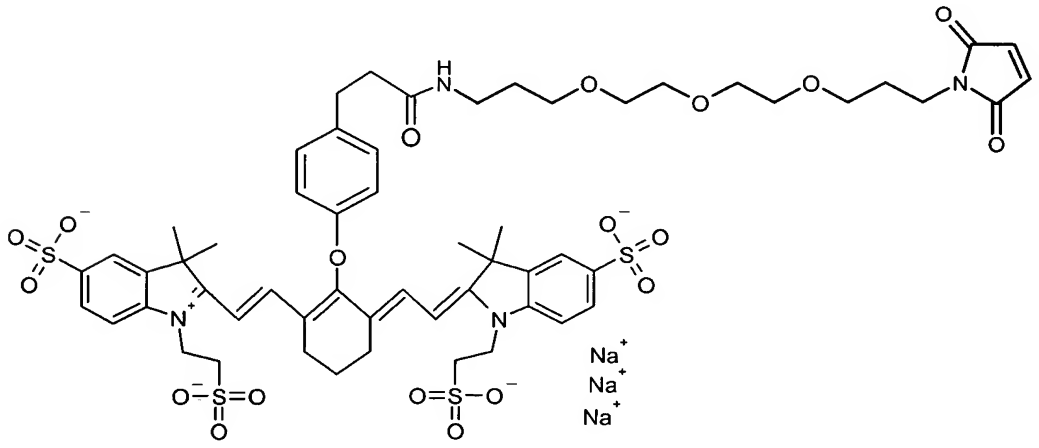


The synthesis is carried out analogously to Example 1e) from 0.7 g (0.68 mmol) of the title compound of Example 14a) and 0.53 g (1.22 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoracetate. Yield: 0.56 g of a blue lyophilizate (68% of theory).

Elementary analysis: Cld.: C 48.27 H 4.53 N 5.36 S 12.27 Na 6.60

Fnd.: C 48.01 H 4.44 N 5.56 S 12.10 Na 6.81

Example 15: Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[13-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1- yl)-4,7,10-trioxatridecyl]carbamoyl}ethyl)phenoxy]cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XVI)

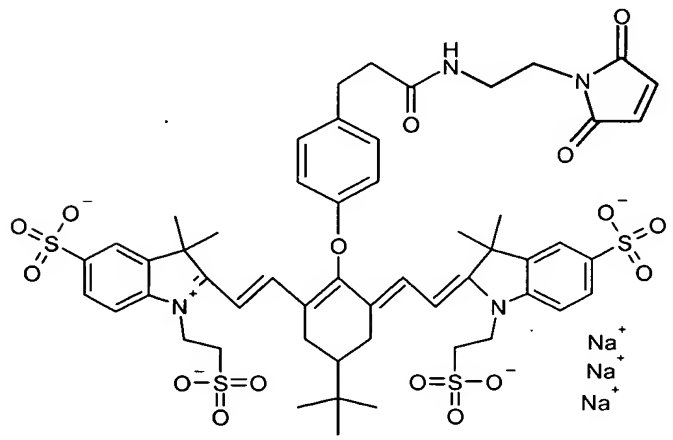


The synthesis is carried out analogously to Example 1e) from 0.7 g (0.68 mmol) of the title compound of Example 14a) and 0.59 g (1.36 mmol) of N-(13-amino-4,7,10-trioxatridecyl)maleimide-trifluoroacetate. Two chromatographic purifications are carried out. Yield: 0.67 g of a blue lyophilizate (75% of theory) .

Elementary analysis: Cld.: C 52.29 H 5.16 N 4.28 S 9.79 Na 5.27

Fnd.: C 51.88 H 5.40 N 4.34 S 9.53 Na 5.68

Example 16: Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1- yl)ethyl]carbamoyl}ethyl)-phenoxy]-5-*tert*-butyl-cyclohex-1-en-3-yliden)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XVII)



a) N-[(3-(Anilinomethylene)-2-chloro-5-*tert*-butyl-1-cyclohexen-1-yl)methylene]aniline hydrochloride

6.7 ml (73.4 mmol) of phosphorus oxychloride is added in drops at 0°C to 8 ml of dimethylformamide. Then, a solution of 5.0 g (32.4 mmol) of 4-*tert*-butylcyclohexanone in 30 ml of dichloromethane is added in drops, and the reaction mixture is stirred under reflux for 3 hours. After cooling to 0°C, 6 g (64.8 mmol) of aniline in 5.5 ml of ethanol is slowly added in drops, the mixture is poured onto 200 g of ice, and 5 ml of concentrated hydrochloric acid is added while being stirred. The precipitated solid is filtered off, washed with ether and dried. Yield: 6.8 g (50% of theory) of a red solid.

b) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-chloro-5-*tert*-butylcyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

5.0 g (14.4 mmol) of 1-(2-sulfonatoethyl)-2,3,3-trimethyl-3H-indolenine-5-sulfonic acid (Example 1a)) and 3.0 g (7.2 mmol) of N-[(3-(anilinomethylene)-2-chloro-5-*tert*-butyl-1-cyclohexen-1-yl)methylene]aniline hydrochloride (Example 16a)) are refluxed together with 2.5 g (30 mmol) of anhydrous sodium acetate in 100 ml of methanol for 1.5 hours, cooled, mixed with 200 ml of diethyl ether and stirred overnight. The precipitate is suctioned off, dried and purified by chromatography (silica gel, gradient: dichloromethane/methanol). Yield: 4.7 g (68% of theory) of a blue solid.

c) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-carboxyethyl)phenoxy]-5-*tert*-butylcyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The reaction is carried out from 2.0 g (2.1 mmol) of the title compound of Example 16b) as described in Example 13b). Yield: 1.5 g (66% of theory).

d) Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl]carbamoyl}ethyl)-phenoxy]-5-*tert*-butyl-cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

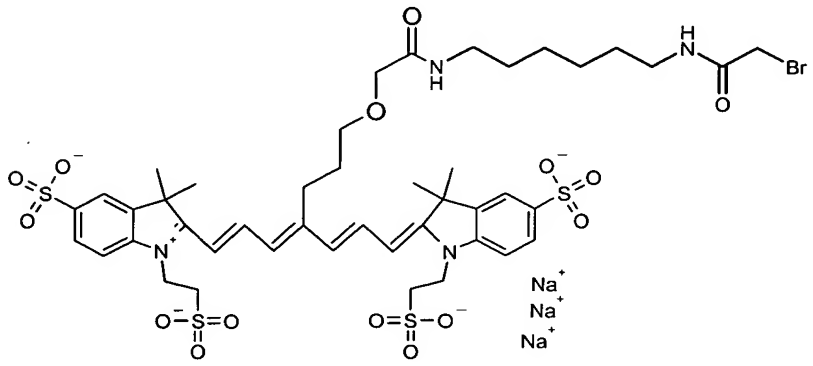
The reaction is carried out from 1.0 g (0.92 mmol) of the title compound of Example 16c) as described in Example 13c). The purification by chromatography is carried out twice with RP C-18 silica gel (mobile solvent: acetonitrile/water). Yield: 0.24 g (22% of theory).

Elementary analysis: Cld.: C 52.82 H 4.93 N 4.65 S 10.64 Na 5.72

Fnd.: C 52.23 H 5.20 N 4.31 S 10.30 Na 6.15

Examples 17 - 19: Synthesis of Indotricarbocyanine Dyes with Bromoacetylamide Groups

Example 17: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[6-(bromoacetylamino)hexyl]carbamoyl}-4-oxapentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XIX)



a) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{(6-aminohexyl)carbamoyl}-4-oxapentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

The synthesis is carried out analogously to Example 1e) from 0.5 g (0.55 mmol) of the title compound of Example 10c) and 0.15 g (0.70 mmol) of N-boc-hexanediamine (*Fluka*). The reaction product is purified by chromatography (RP C18-chromatography, gradient: methanol/water) and after freeze-drying, it is stirred in 2 ml of trifluoroacetic acid/8 ml of dichloromethane for 15 minutes while being cooled with ice. After spinning-in in a vacuum,

the residue is dissolved in methanol, precipitated with diethyl ether and isolated. Yield: 0.26 g of a blue solid (41% of theory) .

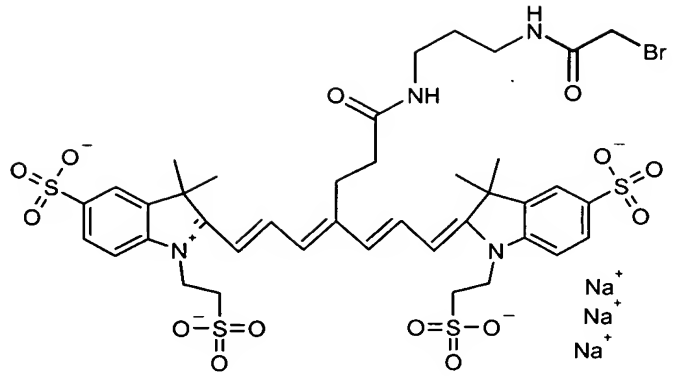
b) Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(5-{[6-(bromoacetylamino)hexyl]carbamoyl}-4-oxapentyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt

0.26 g (0.23 mmol) of the title compound of Example 18a) is cooled in 5 ml of dimethylformamide to -20°C, mixed with 28 mg (0.28 mmol) of triethylamine and a solution of 0.10 g (0.46 mmol) of bromoacetyl bromide in 0.2 ml of dimethylformamide. After 5 hours of stirring at a maximum of 0°C, the product is precipitated by adding diethyl ether and obtained by repeated re-precipitation from dimethylformamide/diethyl ether and subsequent drying. Yield: 0.23 g (86% of theory) of a blue solid.

Elementary analysis: Cld.: C 45.63 H 4.87 N 4.84 S 11.07 Na 5.96

Fnd.: C 45.13 H 4.66 N 4.67 S 10.83 Na not determined

Example 18: Trisodium 3,3-dimethyl-2-{7-[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]-4-(3-{[3-(bromoacetylamino)propyl]carbamoyl}-ethyl)hepta-2,4,6-trien-1-ylidene}-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XVIII)

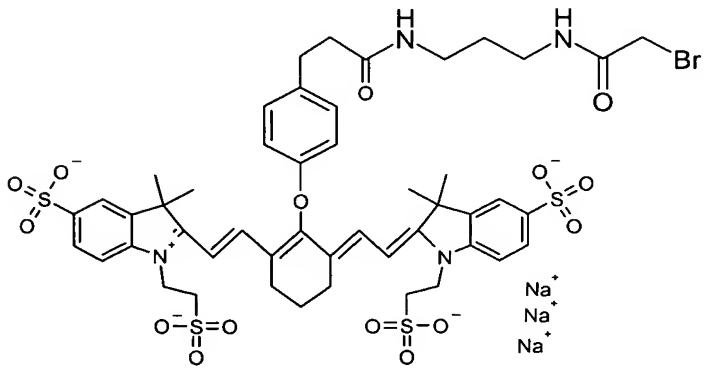


The synthesis is carried out starting from the title compound of Example 1d) (0.5 g; 0.56 mmol) and N-boc-propylenediamine analogously to Example 17. Yield over all the stages: 0.22 g (37% of theory).

Elementary analysis: Cld.: C 43.70 H 4.33 N 5.23 S 11.96 Na 6.43

Fnd.: C 43.21 H 4.14 N 5.53 S 10.89 Na not determined

Example 19: Trisodium 3,3-dimethyl-2-[2-(1-{[3,3-dimethyl-5-sulfonato-1-(2-sulfonatoethyl)-3H-indolium-2-yl]vinylene}-2-[4-(2-{[3-(bromoacetylamino)propyl]carbamoyl}ethyl)-phenoxy]cyclohex-1-en-3-ylidene)ethylidene]-1-(2-sulfonatoethyl)-2,3-dihydro-1H-indole-5-sulfonate, internal salt (Formula XX)



The synthesis is carried out starting from the title compound of Example 13b) (0.5 g; 0.49 mmol) and N-boc-propylenediamine analogously to Example 17. Yield over all stages: 0.31 g (53% of theory).

Elementary analysis: Cld.: C 47.88 H 4.52 N 4.65 S 10.65 Na 5.73

Fnd.: C 48.04 H 4.43 N 4.69 S 10.72 Na 5.84

Examples 20–23: Synthesis of conjugates with biomolecules and photophysical characterization of the conjugates

Example 20: Labeling of BSA (bovine serum albumin) with the title compounds of Examples 1-16

General instructions: A solution of 5 mg (0.074 µmol) of BSA (*Sigma Company*) in 5 ml of phosphate buffer (0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.8) is mixed in each case with 0.74 µmol of the title compounds of Examples 1-16 (stock solutions of 0.5 mg/ml in PBS) and incubated for 30 minutes at 25°C. The purification of the conjugate is carried out by means of gel chromatography (column: Sephadex G50, diameter 1.5 cm, Pharmacia, eluant: PBS).

Example 21: Labeling of BSA with the title compounds of Examples 17-19

General instructions: A solution of 5 mg (0.074 µmol) of BSA (*Sigma Company*) in 5 ml of phosphate buffer (0.1 M borate buffer, pH 8.5) is mixed in each case with 1.10 µmol of the title compounds of Examples 17-19 (stock solutions of 0.5 mg/ml in PBS) and incubated for 5 hours at 25°C. The purification of the conjugate is carried out by means of gel chromatography (column: Sephadex G50, diameter 1.5 cm, Pharmacia, eluant: PBS).

Example 22: Labeling of anti-ED-B-fibronectin scFv antibody AP39 (single chain fragment) with the title compounds of Examples 1-16

AP39 is an scFv with a C-terminal cysteine and is present as a covalent S-S-dimer of the molar-mass of about 56,000 g/mol (Curr Opin Drug Discov Devel. 2002 Mar; 5(2): 204-13). By reduction of the disulfide bridges, two monomers with accessible SH groups are produced (molar mass 28,000 g/mol).

General instructions: 0.3 ml of a solution of AP39 in PBS (conc. 0.93 mg of dimer/ml) is mixed with 60 µl of a solution of tris(carboxyethyl)phosphine (TCEP) in PBS (2.8 mg/ml) and incubated under nitrogen for 1 hour at 25°C. Excess TCEP is separated by means of gel filtration on an NAP-5 column (eluant: PBS). The quantity of AP39-monomer obtained (OD_{280nm} = 1.4), determined by means of photometry, is 230–250 µg (volumes 0.5 – 0.6 ml). The solution is mixed with 0.03 µmol of the title compounds of Examples 1-16 (stock solutions of 0.5 mg/ml in PBS) and incubated for 30 minutes at 25°C. The conjugate is purified by gel chromatography on an NAP-5 column (eluant: PBS/10% glycerol). The immune reactivity of the conjugate solution is determined by means of affinity chromatography (ED-B-fibronectin resin) (*J Immunol Meth 1999, 231, 239*). The immune reactivity of the conjugates obtained was >80% (AP39 before the conjugation >95%).

Example 23: Labeling of anti-ED-B-fibronectin scFv antibodies AP39 (single chain fragment) with the title compounds of Examples 17-19

General instructions: 0.3 ml of a solution of AP39 in PBS (conc. 0.93 mg of dimer/ml) is mixed with 60 µl of a solution of tris(carboxyethyl)phosphine (TCEP) in PBS (2.8 mg/ml) and incubated under nitrogen for 1 hour at 25°C. Excess TCEP is separated by means of gel filtration on an NAP-5 column (eluant: 50 mmol of borate buffer pH 8.5). The quantity of AP39-monomer (OD_{280nm} = 1.4) that is obtained, determined by means of photometry, is

230 – 250 µg (volumes 0.5 – 0.6 ml). The solution is mixed with 0.06 µmol of the title compounds of Examples 17-19 (stock solutions of 0.5 mg/ml in PBS) and incubated for 4 hours at 25°C. The conjugate is purified by gel chromatography on an NAP-5 column (eluant: PBS/10% glycerol). The immune reactivity of the conjugate solution is determined by means of affinity chromatography (ED-B-fibronectin resin) (*J Immunol Meth 1999, 231, 239*). The immune reactivities of the conjugates that were obtained was >75% (AP39 before the conjugation >95%).

Photophysical Characterization of the Dye-BSA-Conjugates of Examples 21 and 22 and the Dye-scFv Antibody Conjugates of Examples 23 and 24.

The degree of concentration (dye/antibody molar ratio) is determined by photometry and based on an extinction coefficient of 75000 L mol^{-1} cm^{-1} in the short-wave absorption shoulder (about 690 – 710 nm); the antibody absorption (AP39) is determined with an OD_{280nm} of 1.4; and/or the protein absorption (BSA) is determined with an OD_{277nm} of 0.58. The fluorescence quantum yield is determined with a SPEX fluorolog (wavelength-dependent sensitivity calibrated by lamp and detector) relative to Indocyanine Green (Q = 0.13 in DMSO, *J Chem Eng Data 1977, 22, 379, Bioconjugate Chem 2001, 12, 44*).

Table 2: Properties of Conjugates According to the Invention

	Substance (Biomolecule/ Sample Compound)	Degree of Concentration	Absorption Maximum (nm)	Fluorescence Maximum (nm)	Fluorescence Quantum Yield
Example 20					
K1	Conjugate from BSA and the title compound of Example 2	0.5	766	790	0.13

	Substance (Biomolecule/ Sample Compound)	Degree of Concen-tration	Absorption Maximum (nm)	Fluorescence Maximum (nm)	Fluorescence Quantum Yield
K2	Conjugate from BSA and the title compound of Example 4	0.6	767	792	0.16
K3	Conjugate from BSA and the title compound of Example 5	0.7	765	790	0.15
K4	Conjugate from BSA and the title compound of Example 10	0.5	766	789	not deter-mined
K5	Conjugate from BSA and the title compound of Example 11	0.5	768	790	0.14
K6	Conjugate from BSA and the title compound of Example 13	0.4	772	793	0.11
K7	Conjugate from BSA and the title compound of Example 16	0.4	772	793	not deter-mined
Example 21					
K8	Conjugate from BSA and the title compound of Example 17	0.3	768	790	0.15
Example 22					
K9	Conjugate from AP39 and the title compound of Example 1	1.1	768	794	0.14
K10	Conjugate from AP39 and the title compound of Example 2	1.0	767	793	0.12
K11	Conjugate from AP39 and the title compound of Example 4	0.8	767	792	0.12

	Substance (Biomolecule/ Sample Compound)	Degree of Concen-tration	Absorption Maximum (nm)	Fluorescence Maximum (nm)	Fluorescence Quantum Yield
K12	Conjugate from AP39 and the title compound of Example 5	0.9	768	794	0.14
K13	Conjugate from AP39 and the title compound of Example 6	1.1	769	792	0.10
K14	Conjugate from AP39 and the title compound of Example 7	1.0	769	792	not deter-mined
K15	Conjugate from AP39 and the title compound of Example 10	1.1	767	790	0.13
K16	Conjugate from AP39 and the title compound of Example 11	1.1	767	789	0.15
K17	Conjugate from AP39 and the title compound of Example 12	0.9	766	790	0.11
K18	Conjugate from AP39 and the title compound of Example 13	1.2	771	795	0.10
K19	Conjugate from AP39 and the title compound of Example 14	1.1	772	796	0.09
Example 23					
K20	Conjugate from AP39 and the title compound of Example 17	0.7	767	790	0.18
K21	Conjugate from AP39 and the title compound of Example 19	0.8	773	794	0.13

Example 24:

The imaging properties of the conjugates according to the invention were examined *in vivo* after injection in tumor-carrying hairless mice. For this purpose, the conjugates were administered intravenously, and the concentration in the tumor region was observed in a period of 0 to 24 hours. The fluorescence of the substances was excited by area irradiation of the animals with near-infrared light with a 740 nm wavelength, which was produced with a laser diode (0.5 W output). The fluorescence radiation was detected by an intensified CCD camera, and the fluorescence images were stored digitally. The *in-vivo* effectiveness of the dye conjugates is depicted in Figure 2 based on an example.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius, and all parts and percentages are by weight, unless otherwise indicated.

The entire disclosures of all applications, patents and publications, cited herein and of corresponding German application No. 103 02 787.4, filed January 24, 2003 and U.S. Provisional Application Serial No. 60/443,197, filed January 29, 2003 are incorporated by reference herein.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.